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Suitability and Clinical Application of a Multiplex Nested PCR Assay for the Diagnosis of Herpes Simplex Virus Infections

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A novel multiplex nested polymerase chain reaction (PCR) assay was designed and evaluated for routine diagnosis of herpes simplex virus (HSV) infections in patients with either putative HSV infection of the central nervous system or suspected HSV keratitis. Single-tube amplification of HSV type 1 (HSV-1) or type 2 (HSV-2) DNA extracted from cerebrospinal fluid (CSF) or from keratectomy specimens was followed by differentiation of the virus type-specific PCR products either by agarose gel analysis or by DNA enzyme immunoassay. Among 417 CSF specimens obtained from 395 consecutive patients with clinically suspected HSV infection, 11 (2.6%) were positive for HSV-1 DNA and four (1.0%) probes were positive for HSV-2 DNA. None of the specimens was positive for both HSV-1 and HSV-2 DNA. The genome of HSV-2 was detected in a CSF sample obtained from a woman with meningoencephalitis and genital herpes. The presence of PCR inhibitors was detected in six of 111 (5.4%) reconstructed CSF samples. Inhibition could be removed following extraction with a commercial kit. HSV-1 DNA, but no HSV-2 DNA, was detected in corneal buttons obtained from patients with suspected herpetic keratitis. No contamination has been recorded during the 2-year routine use of this test, which has met the specific requirements of a diagnostic laboratory.

KEY WORDS: polymerase chain reaction, HSV-1, HSV-2, encephalitis, keratitis, cerebrospinal fluid

INTRODUCTION

Herpes simplex virus (HSV) type 1 and type 2 can cause various types of infection [Whitley, 1996], among which involvement of the central nervous system (CNS) may take a rapid course leading to herpes simplex encephalitis (HSE). HSE has a yearly incidence of 1–4 per million persons [Whitley and Lakeman, 1995; Aurelius, 1993] and may be associated with significant morbidity and a mortality of about 70% without treatment [Mertens et al., 1993; Guffond et al., 1994]. In adults, HSE is most frequently, but not exclusively, caused by infection with HSV-1 [Aurelius et al., 1993; Miller et al., 1995]. Life-threatening CNS infections in the neonate are caused predominantly by HSV-2 [Whitley and Lakeman, 1995; Aurelius, 1993]. In immunocompetent patients older than 3 months, meningitis, meningoencephalitis, and myelitis are often associated with HSV-2 [Shoji et al., 1994; Aurelius, 1993]. Moreover, predominantly HSV-2 may lead to benign recurrent aseptic meningitis, such as Mollaret's meningitis [Cohen et al., 1994; Picard et al., 1993; Tedder et al., 1994].

HSV keratitis, which is primarily produced by HSV-1, is an important viral cause of blindness in industrialized countries [Whitley, 1996; Harding, 1993; Beigi et al., 1994]. Primary HSV corneal infection is frequently followed by recurrent episodes leading to disciform keratitis with scarring and decreased visual acuity.

Early diagnosis of HSE is important to start adequate treatment and to exclude other diseases that have a similar clinical presentation. Clinical diagnosis is often inconclusive due to the lack of pathognomonic clinical features. Upon corneal involvement, the diagnosis of herpes simplex keratitis is difficult to establish in patients presenting with advanced stages of the disease. In cases of either CNS or ocular involvement, specific and rapid diagnosis can be established by direct demonstration of HSV DNA in cerebrospinal fluid (CSF) or keratectomy specimens using polymerase chain reaction (PCR) [Rowley et al., 1990; Puchhammer-Stöckl et al., 1990; Aurelius et al., 1993; Picard et al., 1993; Openshaw et al., 1995; Powell et al., 1990; Anderson et al., 1993; Lakeman et al., 1995].

A new multiplex nested-PCR assay for single-tube am-

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plification of HSV-1 and HSV-2 DNA is described. This test has been developed and evaluated prospectively for routine diagnosis of HSV-1 or HSV-2 infections in the CSF of patients with clinical suspicion of HSV infection of the CNS or in corneas from patients with presumed herpetic eye disease.

MATERIALS AND METHODS

Specimens

CSF specimens (n = 417) were obtained prospectively between the autumn of 1993 and the autumn of 1995 from 395 consecutive patients with clinical suspicion of HSE, meningitis, or meningoencephalitis. Keratectomy specimens (corneal buttons) were obtained after penetrating keratoplasty from patients with suspected herpetic disease.

Controls

Negative controls consisted of CSF collected from patients with unrelated diseases. These were extracted using the same procedure as for the clinical CSF specimens and were subsequently included in each PCR run. Corneal buttons obtained from patients with bullous keratopathy and no history of infectious keratitis were used as negative controls for the keratectomy specimens. Several PCR amplimixes containing no DNA were included in each PCR run as a negative control to monitor potential carry-over contamination. Serial dilutions of titrated suspensions of either HSV-1 strain 539 (a gift of Dr. Puchhammer-Stöckl, Vienna, Austria) or HSV-2 isolated from clinical material (a gift of Dr. D. Schultze, St. Gallen, Switzerland) were used as positive controls.

DNA Extraction From Clinical Specimens

HSV DNA was recovered from CSF according to the method described by Kawasaki [1990]. Briefly, 100 μl of lysis buffer K (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1% Laureth 12 or 0.5% Tween 20, 100 μg/ml fresh proteinase K) were added to 50 μl of CSF sample. Digestion was allowed to proceed by incubation at 56°C for 45 min. The protease was subsequently heat-inactivated.

Following penetrating keratoplasty, corneal buttons were fixed in 4% paraformaldehyde and embedded in paraffin. HSV DNA was extracted from 10 μM thick paraffin sections according to the technique of Wright and Manos [1990] with modifications. Briefly, paraffin was removed by organic extraction with octane, followed by two ethanol washes and rinsing with acetone. HSV DNA was extracted from the dried sample by adding 100 μl of lysis buffer K2 (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1% Laureth 12 or 0.5% Tween 20, 200 μg/ml fresh proteinase K) and incubation at 56°C for 3 hr or at 37°C overnight. The protease was then heat-inactivated.

Primers and Probes

The sequences of primers and probes used for PCR amplification and detection of the products were selected in the UL-42 [McGeoch et al., 1988] and US-4 [McGeoch et al., 1987] gene regions of HSV type 1 and type 2, respectively (Table I). The sequence amplified in the UL-42 region of HSV-1 is similar to that described by Puchhammer-Stöckl and co-workers [1990]. For HSV-2, the sequence amplified within the US-4 gene region (encoding the virion glycoprotein gG-2) is derivated from that previously selected by Aurelius and colleagues [1993]. Primer sequences were selected using primer analysis software for a MacIntosh computer (Oligo, version 4.0; National Biosciences Inc., Plymouth, MN). Primers were synthesized in our laboratory using a 392-DNA synthesizer (Applied Biosystems Inc., Foster City, CA).

Multiplex-Nested PCR Amplification

Two successive sets of amplification were carried out in a GeneAmp PCR system 9600 (Perkin-Elmer, Foster City, CA). Following an initial denaturation step at 93.8°C for 2 min, the first round of amplification consisted of 35 cycles (denaturation at 93.8°C for 48 sec, annealing at 55°C for 18 sec, and elongation at 72°C for 48 sec) using 0.4 μM of each outer primer (HSV-1-D2, HSV-1-R2, HSV-2-D1, HSV-2-R1), 400 μM each of four dNTPs (Perkin-Elmer), 2.5 units of Taq DNA polymerase (Perkin-Elmer), 5 μl of GeneAmp 10× PCR buffer (Perkin-Elmer; 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 0.01% gelatin [w/v]), and 10 μl of the extracted DNA in a total volume of 50 μl. After the first amplification, 10 μl of the PCR reaction was used as substrate for a second round of amplification, with 0.2 μM each of the inner nested primers (HSV-1-D3, HSV-1-R3, HSV-2-D3, HSV-2-R3), 2.5 units of Taq DNA polymerase and 200 μM each of four dNTPs in a final volume of 100 μL. The second round of amplification consisted of 15 cycles performed under the same conditions as the first round, except that annealing occurred at 65°C instead of 55°C. At 65°C, preferential amplification of the inner nested fragments is favored for both HSV-1 and HSV-2 sequences.

Detection of PCR Products

After completion of the second amplification run, detection and differentiation of virus type-specific PCR products were carried out either by agarose gel analysis or by DNA enzyme immunoassay (DEIA). For gel electrophoresis, 15 μl of amplicons were loaded on a 3% agarose gel (Nusieve 3:1, FMC Corp., Rockland, ME) stained with ethidium bromide. The sizes of the diagnostic fragments amplified with the inner primers HSV-1-D3/HSV-1-R3 and HSV-2-D3/HSV-2-R3 were 159 base pairs (bp) and 225 bp for HSV-1 and HSV-2, respectively. The DEIA (Gen-Eti-K DEIA; Sorin Biomedica, Saluggia, Italy) is based on microtiter plate hybridization of PCR products with HS15 [Puchhammer-Stöckl et al., 1990] or HSV-2-P30, which are single-stranded internal probes specific for HSV-1 or HSV-2, respectively (Table I). Hybrids were detected spectrophotometrically at 450 nm (OD_{450}) following binding with a mouse anti-DNA monoclonal antibody specific for double-stranded DNA. The

TABLE I. Primers and Probes Used for HSV-1 and HSV-2 Multiplex Nested-PCR

Name	Function	Sequence (5′ to 3′)	Specificity	Gene region	5′ extremity at NT
HSV-1-D2	Outer direct primer	GCT TTG TGG TGC TGG TT	HSV-1	UL-42[a]	93′603
HSV-1-R2	Outer reverse primer	CTG GTG CTG GAC GAC AC	HSV-1	UL-42	93′810
HSV-1-D3	Inner direct primer	CCC CGA CGT TCA GTT GCG CCT GAC G	HSV-1	UL-42	93′631
HSV-1-R3	Inner reverse primer	TCC TCG CGG GCA GCA AAG GTG ACG C	HSV-1	UL-42	93′789
HS15[b]	Probe	ATA GTG CCA CGC CCA CCA CGT TCG A	HSV-1	UL-42	93′696
HSV-2-D1	Outer direct primer	ACG TAC TAC CGG CTC AC	HSV-2	US-4[c]	3′175
HSV-2-R1	Outer reverse primer	CCA CCT CTA CCC ACA AC	HSV-2	US-4	3′433
HSV-2-D3	Inner direct primer	CCG CGC CTG CCG TCA GCC CAT CCT C	HSV-2	US-4	3′192
HSV-2-R3	Inner reverse primer	AGA CCC ACG TGC AGC TCG CCG	HSV-2	US-4	3′416
HSV-2-P30	Probe	CCC TGC TGG TGC CGA TCT GGG ACC GCG CCG	HSV-2	US-4	3′335

[a]McGeoch et al. [1988].
[b]Puchhammer-Stöckl et al. [1990].
[c]McGeoch et al. [1987].

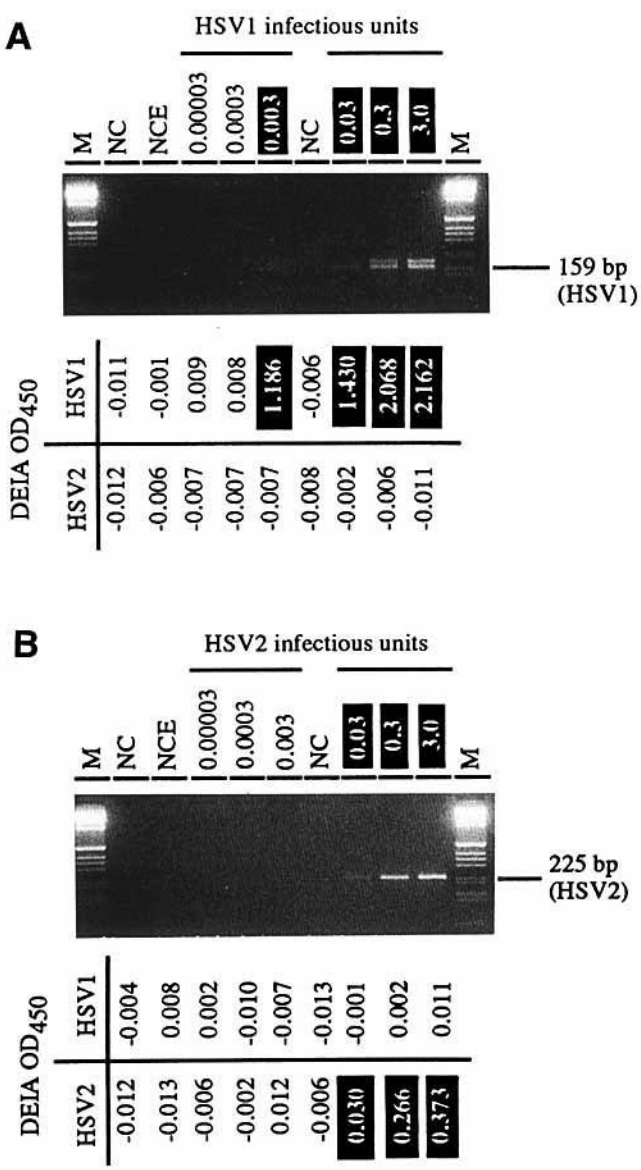

Fig. 1. Specificity and sensitivity of the HSV-1/HSV-2 multiplex nested-PCR protocol. Serial dilutions of known titers of HSV-1 **(A)** or HSV-2 **(B)** were done in CSF. Up to 0.003 tissue culture infectious units of HSV-1 and 0.03 infectious units of HSV-2 can be detected by agarose gel analysis or DEIA. M, marker; NC, negative control; NCE, negative control extracted like diagnostic probes; bp, base pairs; HSV1, bound probe (HS15) specific for HSV-1; HSV2, bound probe (HSV-2-P30) specific for HSV-2; DEIA OD_{450}, optical density at 450 nm for the microtiter plate assay.

cut-off value was set at $OD_{450} = 0.030$ for both HSV-1 and HSV-2.

RESULTS

Sensitivity and Specificity of the PCR Assay

The sensitivity of the multiplex nested PCR assay was determined using 10-fold serial dilutions of cells in culture infected with either HSV-1 or HSV-2 (Fig. 1). Dilutions were carried out in CSF obtained from patients with unrelated infections in order to use the same biological material as for the diagnostic PCR. Following agar-

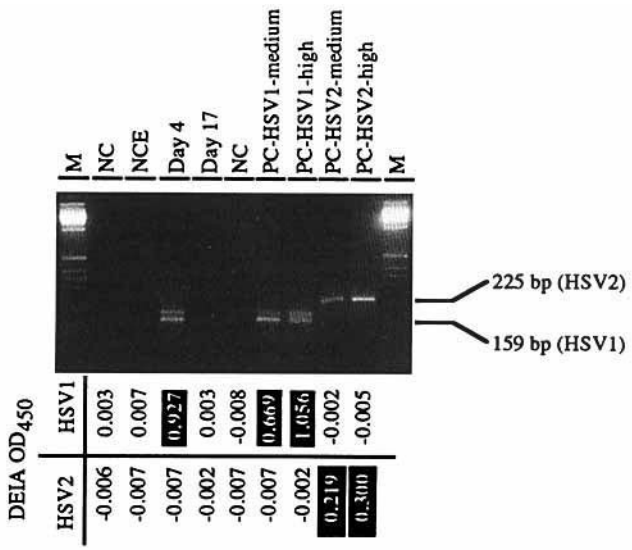

Fig. 2. Herpes simplex encephalitis in a 71-year-old man. Detection of HSV-1 DNA by PCR in a CSF sample collected 4 days after onset of clinical symptoms (lane "Day 4"). Neither HSV-1 nor HSV-2 DNA were present in a second CSF sample obtained 13 days later (Lane "Day 17"). PCR products were analyzed by agarose gel electrophoresis and DEIA. M, marker; NC, negative control; NCE, negative control extracted like diagnostic probes; bp, base pairs; HSV1, bound probe (HS15) specific for HSV-1; HSV2, bound probe (HSV-2-P30) specific for HSV-2; DEIA OD_{450}, optical density at 450 nm for the microtiter plate assay; PC-HSV1-medium, positive control corresponding to 0.3 tissue culture infectious units of HSV-1; PC-HSV1-high, positive control corresponding to 3.0 tissue culture infectious units of HSV-1; PC-HSV2-medium, positive control corresponding to 0.3 tissue culture infectious units of HSV-2; PC-HSV2-high, positive control corresponding to 3.0 tissue culture infectious units of HSV-2.

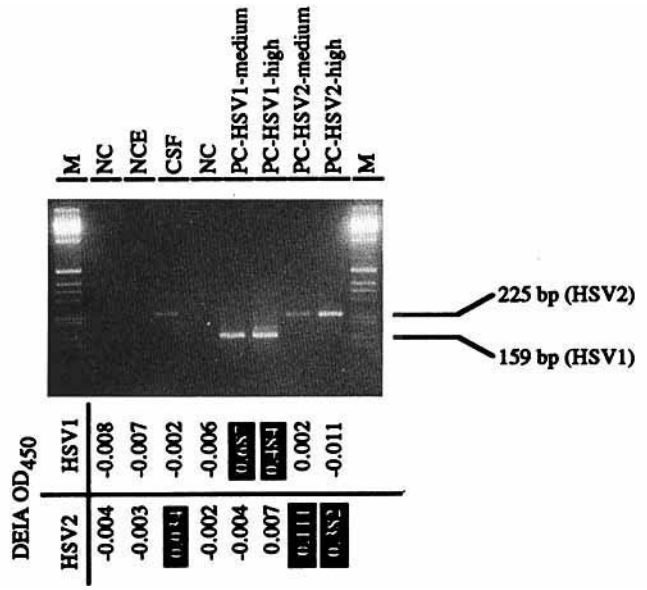

Fig. 3. Meningoencephalitis in a 22-year-old woman with herpes genitalis. HSV-2 DNA was detected by PCR in a CSF sample obtained shortly after onset (Lane "CSF"). PCR products were analyzed by agarose gel electrophoresis and DEIA. The abbreviations are the same as in Figure 2.

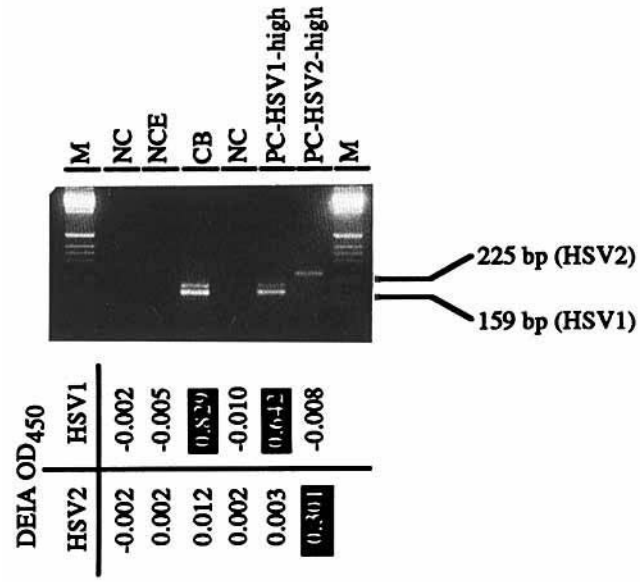

Fig. 4. Herpes simplex keratitis. Detection of HSV-1 DNA by PCR in a corneal button (Lane "CB") obtained during penetrating keratoplasty. PCR products were analyzed by agarose gel electrophoresis and DEIA. The abbreviations are the same as in Figure 2.

ose gel electrophoresis, the minimal amounts of HSV-1 and HSV-2 detectable were 0.003 and 0.03 tissue culture infectious units, respectively. The same detection limit was obtained by DEIA, i.e., following hybridization of the PCR product and colorimetric detection in a microtiter plate (Fig. 1). Subsequently, serial dilutions of HSV-1 and HSV-2 ranging from 3.0 to 0.03 infectious units were used as positive controls to monitor the sensitivity of each assay in routine application.

The specificity of the PCR protocol for either HSV-1 or HSV-2 was demonstrated by amplicons of 159 bp and 225 bp, respectively (Fig. 1). The identity of the PCR products and the absence of cross-reaction between HSV-1 and HSV-2 was further confirmed by DEIA. No signal was obtained by DEIA following detection of amplicons resulting from HSV-1 using the HSV-2 specific probe or, reciprocally, following detection of amplicons resulting from HSV-2 using the HSV-1-specific probe (Fig. 1).

An additional PCR product of larger size (approximately 180 bp) is visible on the gel at high concentration of HSV-1. This amplicon of intermediate length is specific for HSV-1. It results from amplification with a combination of the inner and outer primers which occurred despite the increased annealing temperature in the second round of amplification.

The specificity of primers was evaluated further using other herpes viruses. No amplification occurred with cytomegalovirus (CMV) or varicella-zoster virus (VZV) used as substrate (data not shown).

PCR Detection of HSV Type 1 or Type 2 DNA in Clinical Specimens

Figures 2–4 describe different clinical situations where an HSV infection was diagnosed by PCR.

TABLE II. Detection of HSV-1 and HSV-2 Genome by Multiplex Nested-PCR in 417 CSF Samples Obtained From 395 Patients With Clinical Suspicion of CNS Herpetic Disease

HSV-1-PCR	HSV-2-PCR	Number of samples (%)	Number of patients (%)
Negative	Negative	402 (96.4)	384 (97.2)
Positive	Negative	11 (2.6)	8 (2.0)
Negative	Positive	4 (1.0)	3 (0.8)
Positive	Positive	0 (0.0)	0 (0.0)
Total		417 (100.0)	395 (100.0)

The first case concerns a 71-year-old man who was admitted to the hospital with clinical suspicion of herpes encephalitis (Fig. 2). HSV-1 DNA, but no HSV-2 DNA, was detected by PCR in a CSF probe collected 4 days after the beginning of symptoms (Fig. 2, lane "day 4"). A second CSF sample collected 17 days after the onset of symptoms tested negative for both HSV-1 and HSV-2 DNA (Fig. 2, lane "day 17"). At that time, however, the diagnosis of HSE could be established by demonstration of intrathecal synthesis of anti-HSV antibody (data not shown). In contrast, no elevated titer of anti-HSV antibody could be evidenced in the first CSF sample (data not shown).

Detection of the HSV-2 genome by PCR in a 22-year-old woman with herpes genitalis and clinical suspicion of menigoencephalitis is presented in Figure 3. HSV-2 DNA was detected by PCR in a CSF sample obtained 3 days after the onset of symptoms (Fig. 3, lane "CSF").

Ocular infection with HSV type 1 can cause disciform herpetic keratitis. The clinical diagnosis of HSV-associated keratitis can be confirmed by PCR (Fig. 4). HSV-1 DNA, but no HSV-2 DNA, was detected following PCR amplification in a corneal button obtained during penetrating keratoplasty from a patient with clinical suspicion of herpetic disease (Fig. 4, lane "CB"). The clinical use of multiplex nested-PCR to investigate the presence of HSV-1 and HSV-2 DNA in keratectomy specimens has been evaluated separately [Mietz et al., 1995]. In addition, the diagnosis of herpes keratitis could be established in a 38-year-old man who presented with herpes labialis, reduced corneal sensitivity, and decreased visual acuity of the left eye, by demonstration of HSV-1 DNA using PCR in a corneal swab sample obtained 1 week after the beginning of ocular symptoms (data not shown).

PCR Analysis of CSF Samples Obtained From Patients With Clinical Suspicion of HSV Encephalitis or Meningitis

The presence of the HSV-1 or HSV-2 genome was investigated by multiplex nested-PCR in a total of 417 CSF specimens obtained prospectively from 395 consecutive patients with clinical suspicion of meningitis or encephalitis (Table II). These samples were sent to our laboratory for routine diagnostic purposes between 1993 and 1995. Whereas 11 samples obtained from eight patients tested positive for HSV-1 DNA, the genome of HSV-2 could be detected in only four samples collected from three patients. No samples were positive for both HSV-1 and HSV-2 DNA.

TABLE III. Investigation of the Presence of PCR Inhibitors in Spiked CSF Specimens

Inhibition HSV-1-PCR	Inhibition HSV-2-PCR	CSF samples tested (%)
No	No	105 (94.6)
Yes	No	0 (0)
No	Yes	1 (0.9)
Yes	Yes	5 (4.5)
		Total: 111 (100)

Potential Presence of Inhibiting Factors in CSF

The presence in CSF of factors able to inhibit the PCR reaction may potentially lead to false-negative results. To judge the extent of this problem, 111 CSF samples shown during previous testing to contain neither HSV-1 nor HSV-2 DNA were spiked with three infectious units each of HSV-1 and HSV-2. DNA was extracted from the spiked specimens and submitted to PCR amplification prior to agarose gel analysis (Table III). The lack of PCR products visible on the gel was taken as being indicative of the presence of inhibitors in the corresponding CSF sample. From a total of 111 CSF samples spiked, the expected PCR products diagnostic for HSV-1 or HSV-2 were obtained in 105 samples (94.6%). Five samples showed inhibition of both HSV-1 and HSV-2, and one sample showed inhibition of HSV-2 only. Inhibition could be removed following extraction of another aliquot with a commercial kit (QIAamp blood kit; Qiagen AG, Basel, Switzerland).

DISCUSSION

Severe HSV infections of the CNS as well as ocular HSV infections potentially causing blindness require early and accurate diagnosis. The suitability of PCR for this purpose has been the subject of many reports [Pohl-Koppe et al., 1992; Guffond et al., 1994; Anderson et al., 1993; Aurelius et al., 1993; Mertens et al., 1993; Picard et al., 1993; Cohen et al., 1994; Tedder et al., 1994; Shoji et al., 1994; Powell et al., 1990; Puchhammer-Stöckl et al., 1990; Rowley et al., 1990; Yamamoto et al., 1994; Openshaw et al., 1995; Miller et al., 1995]. In a recent study, Lakeman and co-workers [1995] have shown that 98% of patients with biopsy-proven HSE had HSV DNA detectable in CSF by PCR, thus highlighting the rele-

vance of PCR for the early diagnosis of HSE when a therapeutic decision is urgent.

The novel multiplex nested-PCR assay was developed for routine application, and its practicability and dependability were then evaluated prospectively over a period of 2 years on CSF samples obtained from patients with clinical suspicion of herpetic CNS disease. A multiplex format allowing simultaneous amplification of HSV-1 and HSV-2 DNA within the same tube has been chosen in order to save labor and to reduce costs. Differentiation between HSV-1 and HSV-2 was required because the extent of the clinical manifestations and, hence, the prognosis of the course of disease is often type-specific. In addition, differentiation between HSV-1 and HSV-2 yields valuable epidemiological information.

Quality controls are of crucial importance to monitor the potential occurrence of false-positive as well as of false-negative PCR results [Landry, 1995]. The identity of HSV-1 or HSV-2 PCR products was revealed upon agarose gel analysis and confirmed by hybridization and colorimetric detection in a microtiter plate format. Potential contaminations leading to false-positive results were monitored by submitting control amplimixes containing no DNA to the whole PCR process, including DNA extraction.

In addition to false-positive results, the PCR process is likely to yield false-negative results caused by the presence of inhibitors of the DNA polymerase [Wiedbrauk et al., 1995; Cone et al., 1992]. Due to the profound clinical and therapeutic implications of a false-negative PCR result, identification of inhibited PCR reactions is a priority. Using reconstructed specimens, a 5.4% inhibition rate was detected. Inhibitors could be eliminated by DNA extraction with a commercial kit. In routine practice, the presence of inhibitors was subsequently assessed by testing a distinct aliquot of each clinical specimen spiked with HSV-1 and HSV-2.

The multiplex nested-PCR assay has been shown to be appropriate for early detection of HSV infection in patients with herpetic CNS disease suspected on clinical signs before intrathecal synthesis of HSV antibody becomes detectable. Demonstration of HSV-2 DNA in the CSF of a woman with genital herpes and meningoencephalitis, a common complication of genital HSV infection [Whitley, 1996], allowed appropriate therapeutic intervention. Coinfection with HSV-1 and HSV-2 has not been detected.

Demonstration of HSV-1 DNA in corneal buttons and in a corneal swab specimen further illustrates the use of the multiplex nested-PCR assay to diagnose HSV infection in patients with suspected HSV keratitis. Swab specimens collected using a noninvasive procedure may be useful for the diagnosis of herpes keratitis. However, the clinical significance of a positive PCR result remains to be fully appreciated.

In summary, the robustness of the multiplex nested-PCR assay presented in this study meets the requirements of a clinical diagnostic laboratory for routine detection of HSV DNA in clinical specimens from patients with putative HSV infection of the CNS. In the future, the characteristics of PCR diagnostics together with the availability of CSF specimens should allow us to investigate in more detail the full spectrum of clinical manifestations associated with HSV infections of the CNS.

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